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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 31/00

A2

(11) International Publication Number: **WO 99/61010**

(43) International Publication Date: 2 December 1999 (02.12.99)

(21) International Application Number: PCT/EP99/03138

(22) International Filing Date: 7 May 1999 (07.05.99)

(30) Priority Data:
98250177.7 26 May 1998 (26.05.98) EP

(71) Applicant *(for all designated States except US)*: SCHERING AKTIENGESELLSCHAFT [DE/DE]; D–13342 Berlin (DE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: HEGELE–HARTUNG, Christa [DE/DE]; Wöllenbeck 101, D–45470 Mülheim/Ruhr (DE). CAM, Quoc–Lam [DE/DE]; Am Schäfersee 15A, D–13407 Berlin (DE).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: TREATMENT OF INFERTILITY WITH cAMP–INCREASING COMPOUNDS ALONE OR IN COMBINATION WITH AT LEAST ONE MEIOSIS–STIMULATING COMPOUND

(57) Abstract

The present invention relates to a pharmaceutical composition comprising cAMP–increasing compounds in low dose and at least one meiosis–stimulating compound for the treatment of infertility and to the use of low dose cAMP–increasing compounds alone or in combination with at least one meiosis–stimulating compound to increase the rate of fertilization in a mammal and for the preparation of medicaments.

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Treatment of Infertility with cAMP-increasing Compounds alone or in combination with at least one Meiosis-stimulating Compound

The present invention relates to a pharmaceutical composition and its use to treat infertility.

Meiosis is the unique and ultimate event of germ cells on which sexual reproduction is based. Meiosis comprises two meiotic divisions. During the first division, exchange between maternal and paternal genes take place before the pairs of chromosomes are separated into the two daughter cells. These contain only half the number (1n) of chromosomes and 2c DNA. The second meiotic division proceeds without a DNA synthesis. This division therefore results in the formation of the haploid germ cells with only 1c DNA.

The meiotic events are similar in the male and female germ cells, but the time schedule and the differentiation processes which lead to ova and to spermatozoa differ profoundly. All female germ cells enter the prophase of the first meiotic division early in life, often before birth, but all are arrested as oocytes later in the prophase (dictyate state) until ovulation after puberty. Thus, from early life the female has a stock of oocytes which is drawn upon until the stock is exhausted. Meiosis in females is not completed until after fertilization, and results in only one ovum and two abortive polar bodies per germ cell. In contrast, only some of the male germ cells enter meiosis from puberty and leave a stem population of germ cells throughout life. Once initiated, meiosis in the male cell proceeds without significant delay and produces 4 spermatozoa.

Only little is known about the mechanisms which control meiosis in the male and in the female. In the oocyte, recent studies indicate that follicular purines, such as hypoxanthine, and adenosine, could be responsible for meiotic arrest (Downs, S.M. et al. (1985), Dev. Biol. 82: 454-458, Eppig, J.J. et al. (1986) Dev. Biol. 119: 313-321, Downs, S.M. et al. (1993), Mol. Reprod. Dev. 35: 82-94). These purine bases were found in follicular fluid in millimolar concentrations (Eppig, J.J. et al. (1985) Biol. Reprod. 33: 1041-1049). However, the purine bases induced arrest was reversible. This was

provided by experiments in which mice and human oocytes were maintained in meiotic arrest for 24 hours with hypoxanthine followed by a 16-30 hour culture in inhibitor-free medium (Downs, S.M. et al. (1986) Gamet Res. 15: 305-316, Cha, K.Y. et al. (1992) Reprod. Fertil. Dev. 4: 695-701). Nearly 100% of the arrested mice oocytes resumed maturation and, furthermore, the mature oocytes were successfully fertilized and demonstrated complete pre-and post-implantation development. These data collectively support the idea that purines such as hypoxanthine and adenosine are physiologically important in the mechanisms controlling meiotic arrest *in vivo*.

Cyclic adenosine 5'-monophosphate (cAMP) plays a pivotal role as a second messenger in the signal transduction pathway during meiosis in the oocyte. cAMP is generated by the action of adenylate cyclase (AC). cAMP is degraded by the family of phosphodiesterase enzymes (PDE), which produces inactive second messenger products. Hypoxanthine is an inhibitor of cAMP PDE (Eppig, J.J. et al. (1985) Biol. Reprod. 33: 1041-1049). As such, it can prevent the hydrolysis of oocyte cAMP and thereby maintain elevated levels of cAMP in the oocyte. In addition to hypoxanthine, agents acting upstream or downstream of cAMP are able to increase cAMP levels. By this way activation of AC with forskolin, inhibition of PDE with the nonselective 3-isobutyl-1-methylxanthine (IBMX) or inhibition of the oocyte-specific isoform PDE3 with a specific PDE3-inhibitor, e.g. milrinone, leads to meiotic arrest by maintaining elevated levels of c-AMP within the oocytes (Downs SM and Hunzicker-Dunn M (1995) Dev Biol 172: 72-85; Tsafiri A et al. (1996) Dev Biol 178: 393-402).

A PDE3 specific inhibitor has been described as a contraceptive agent (WO98/10765).

The presence of a diffusible meiosis regulating substance was first described (Byskov, A.G. et al. (1976) Dev. Biol. 52: 193-200) in the fetal mouse gonades. A meiosis activating substance (MAS) was secreted in the fetal mouse ovary in which meiosis was ongoing, and a meiosis preventing substance (MPS) was released from the morphologically differentiated testis with resting, non-meiotic germ cells. It was therefore suggested that the relative concentrations of MAS and MPS regulated the beginning, arrest and resumption of meiosis in the male and in the female germ cells (Byskov, A.G. and Høyer P.E. (1994), The Physiology of Reproduction, Knobil, E. and Neill,J.D. (eds.), Raven Press, New York, pp 487-540). A recent article (Byskov, A.G. et al. (1995) Nature 374: 559-562) describes the isolation of certain sterols from

preovulatory ovarian follicular fluid, defined as FF-MAS, and bull testicular testis, defined as T-MAS, that activate oocyte meiosis. This was confirmed by Grøndahl et al. (1998, Biol. Reprod. in press) showing that de novo synthesized FF-MAS is capable of mediating resumption of meiosis in mice oocytes.

Although the use of MAS improves the rate of fertility compared to the currently applied methods, it is still desirable to increase this rate even further.

This is achieved by the present invention which provides a pharmaceutical composition comprising c-AMP-increasing compounds in low dose and at least one meiosis-stimulating compound for the treatment of infertility in mammals, particulary in humans, more particulary in females. The increase of fertility by low dose c-AMP-increasing compounds is surprising since the teaching of the prior art is that c-AMP-increasing compounds are responsible for meiotic arrest and a PDE3 specific inhibitor has even been used as a contraceptive agent. The combination of the low dose cAMP-increasing compounds with at least one meiosis-stimulating compound shows a significantly higher stimulation rate than the meiosis-stimulating compound(s) alone.

In a further embodiment the invention relates to the use of low dose c-AMP-increasing compounds alone or in combination with at least one meiosis-stimulating compound as active substances for the production of a pharmaceutical composition for the treatment of infertility in mammals, particular in humans, more particularly in females. Low dose c-AMP-increasing compounds alone increase the fertilization rate compared to the control where no compounds were added and the combination of c-AMP-increasing compounds in a low dose with a meiosis-stimulating compound shows an even higher rate of fertilization than the meiosis-stimulating compound alone or the low dose c-AMP-increasing compound alone.

In a still further embodiment the invention relates to the use of low dose c-AMP-increasing compounds alone or in combination with at least one meiosis-stimulating compound to increase the rate of fertilization in a mammal, particularly in humans, more particularly in females. This use may be to regulate the fertilization rate in a fertilization culture media.

In another aspect, the invention includes the use of a low dose c-AMP-increasing compounds alone or in combination with at least one meiosis-stimulating compound

for the administration to a germ cell. The germ cell may be an oocyte or a spermatozoon.

In another embodiment the invention relates to a method of stimulating meiosis in a mammalian germ cell comprising administering *ex vivo* or *in vivo* or *in vitro* to a germ cell in need of such a stimulation an effective amount of low dose c-AMP-increasing compounds alone or in combination with at least one meiosis-stimulating compound. The germ cell may be an oocyte or a spermatozoon.

In a further embodiment the invention relates to a pharmaceutical kit comprising a dosage unit for a c-AMP-increasing compound in low dose and a dosage unit of at least one meiosis-stimulating compound. The c-AMP-increasing compound and the meiosis-stimulating compound may be provided in the same application form or in different application forms. Application forms means e.g. tablets, liquid compositions for injections, paste and others well known in the art.

Meiosis-stimulating compounds according to the present invention are all compounds that can activate meiosis. Compounds being known to stimulate meiosis and their synthesis are described, i.e., in WO 96/27658, WO97/00884, WO96/00235, WO98/28323 and WO98/52965. In preferred embodiments of all modes of the invention the meiosis-stimulating compound is FF-MAS (4,4-dimethyl-5α-cholesta-8,14,24-trien-3ß-ol).

Under a significantly higher stimulation rate of fertilization according to the present invention it is meant that the stimulation rate is at least 40 – 50 %, preferred 50 – 75%, and more preferred 75 – 100 %.

Low dose cAMP-increasing compounds according to the present invention is a dose of cAMP-increasing compounds that lead to meiotic maturation without inducing meiotic arrest. In preferred embodiments of the present invention the cAMP-increasing compounds are applied in a dose below 3mM, more preferred in a dose of 0.003 – 1mM, and especially preferred in a dose of 0.1-0.5mM.

In specially preferred embodiments of all modes of the invention the cAMP-increasing compounds are purines, unspecific PDE-inhibitors, specific PDE_3-inhibitors or synthetic membrane permeable cAMP. A purine is e.g. hypoxanthine or adenosine. Unspecific PDE-inhibitors are nonselective inhibitors which inhibit all

types of PDEs, e.g. IBMX, theophylline or SQ20,006 (1-ethyl-4-hydrazino-14-pyrazolo-(3,4-b)-pyridine-5-carboxylicacidethylester). Examples of specific PDE3 inhibitors are milrinone, cilostamide, amrinone, enoximone, lixazinone, indolidan and other inhibitors as listed in WO98/10765. This reference also gives the methods of preparation of these compounds. An example for a synthetic membrane permeable cAMP is dibutyryl-c-AMP (dbcAMP).

In a further specially preferred embodiments of all modes of the invention the meiosis-stimulating compound is FF-MAS and the cAMP-increasing compound is a purine, preferably hypoxanthine.

As described above the present invention relates in further embodiments to pharmaceutical compositions. The compositions may comprise pharmaceutically acceptable excipients well known in the art like carriers, diluents, absorption enhancers, preservatives, buffers, agents for adjusting the osmotic pressure, tablet disintegrating agents and other ingredients which are conventionally used in the art. Examples of solid carriers are magnesium carbonate, magnesium stearate, dextrin, lactose, sugar, talc, gelatin, pectin, tragacanth, methylcellulose, sodium carboxymethyl cellulose, low melting waxes and cacao butter.

Liquid compositions include sterile solutions, suspensions and emulsions. Such liquid compositions may be suitable for injection or for use in connection with *ex vivo*, *in vivo* ,and *in vitro* fertilization. The liquid compositions may contain other ingredients which are conventionally used in the art, some of which are mentioned in the list above. Further, a composition for transdermal administration of a compound of this invention may be provided in the form of a patch, a composition for nasal administration may be provided in the form of a nasal spray in liquid or powder form and a composition for intra-vaginal administration may be provided in the form of a tampon or other intra-vaginal devices.

The dosage to be administered depends to a large extent on the condition and the size of the subject being treated as well as the frequency of treatment and the route of administration.

In general, the compositions of the invention are prepared by intimately bringing into association the active compound or compounds with the liquid or solid auxiliary ingredients and then, if necessary, shaping the product into the desired formulation.

The entire disclosure of all applications, patents and publications, cited above and below are hereby incorporated by reference.

The present invention will be illustrated in detail in the following examples. These examples are included for illustrative purposes and should not be considered to limit the present invention.

Examples

Example 1: Test of hypoxanthine, FF-MAS and the combination of FF-MAS and hypoxanthine in the oocyte assay

Material and Methods:

Naked oocytes (NO) and cumulus enclosed oocytes (CEO) were isolated from follicles from immature (C57B1/6J x DBA/2J)F_1 mice (age 21-24 days), that had received 10 I.U. PMSG i.p. 48h prior to collection. The oocytes were cultured in 4-well multidishes in a modified α-MEM medium containing 1mg fetuin/ml culture medium. Each well contained 0.4 ml of the oocyte culture medium and 35-45 oocytes. The control and test cultures were made with different concentrations of the compounds to be tested as indicated in the tables.

The cultures were kept at 37°C and 100% humidity with 5% CO_2 in the air for 18 hours.

Oocytes arrested in meiosis are characterised by an intact nucleus with a prominent nucleolus, known as germinal vesicle (GV). Upon reinitiation of meiosis the nucleolus and the nuclear envelope disappear and this is characterised by a breakdown of the GV, which than is called germinal vesicle breakdown (GVB).

Results:

Table 1: Activation of meiosis in oocytes using FF-MAS, low dose hypoxanthine and the combination of FF-MAS + hypoxanthine

Compounds	Oocyte type	n Oocytes	GVB (%)
Control (no compounds added)	NO	187	95.1
0.4mM Hx	NO	190	95.8
3mM Hx	NO	170	12.4
10µM FF-MAS + 0.4mM Hx	NO	172	98.3
10µM FF-MAS + 3mM Hx	NO	223	92.3

Hx = Hypoxanthine
NO = naked oocytes
GVB = germinal vesicle breakdown
n = number of

The hypoxanthine used was obtained from Sigma, Deisenhofen, Deutschland. Similar to the control, low dose hypoxanthine (0.4mM) is able to lead to meiotic maturation in most oocytes. However, 3mM hypoxanthine nearly completely prevents meiotic maturation. It is evident that FF-MAS not only when given together with a meiosis – inhibiting dose of hypoxanthine (3mM), but also when given together with low dose hypoxanthine (0.4mM) is able to lead to meiotic maturation in nearly all oocytes.

Example 2: Test of hypoxanthine, FF-MAS and the combination of FF-MAS and hypoxanthine in the in-vitro fertilization (IVF) assay

Material and Methods:

NO and CEO from immature mice (C57B1/6J x DBAJ/2)F_1 were isolated and cultured under the same conditions as described for the oocyte assay. After 18 hours oocytes that exhibited germinal vesicle breakdown (GVB) were shortly washed in hypoxanthine-free medium and transferred to the insemination dishes prepared in advance, which consisted of a motile sperm preparation from the caudal epididymis of male mice. The dishes were then incubated under defined gas conditions (5% CO_2) at 37°C in a modified α-MEM IVF-medium. Neither the insemination medium nor the IVF-medium contained hypoxanthine. Examination of the oocytes were carried out 20-22 hours after insemination, in order to check fertilization and to record the number of 2-cell embryos. The percentage fertilization (= fertilization rate) was determined from counts of oocytes that had cleaved into two-cell embryos.

Results:

Table 2: In vitro fertilization rate (IVF-rate) of oocytes cultured with FF-MAS, low dose hypoxanthine and the combination of FF-MAS + hypoxanthine

Compounds	Oocyte type	n / GVB oocytes used after in vitro culture	Two-cell embryos (%)
Control (no compound added)	NO	177	34.1
0.4mM Hx	NO	182	52.2
3mM Hx	NO	21	0
10µM FF-MAS + 0.4mM Hx	NO	167	64.7
10µM FF-MAS + 3mM Hx	NO	102	51.0

Hx = hypoxanthine

NO = naked oocytes

n = number of

GVB = germinal vesicle breakdown

The hypoxanthine used was obtained from Sigma, Deisenhofen, Deutschland. Oocytes cultured in the presence of 3mM hypoxanthine were unable to get fertilized. 0.4mM hypoxanthine and 10µM FF-MAS + 3 mM hypoxanthine increased the fertilization rate of the control group by about 50%. However, the combination of 10µM FF-MAS + 0.4 mM hypoxanthine could nearly double the control IVF-rate.

Claims

1. A pharmaceutical composition comprising c-AMP-increasing compounds in low dose and at least one meiosis-stimulating compound for the treatment of infertility in mammals.

2. Pharmaceutical composition according to claim 1 wherein the mammal is a human.

3. Pharmaceutical composition according to one of the claims 1 or 2 wherein c-AMP-increasing compounds are purines, unspecific PDE-inhibitors, specific PDE_3-inhibitors and synthetic membrane permeable cAMP.

4. Pharmaceutical composition according to one of the claims 1, 2 or 3 wherein the meiosis-stimulating compound is FF-MAS.

5. Pharmaceutical composition according to one of the claims 1-4 wherein the c-AMP-increasing compound is hypoxanthine.

6. Use of low dose c-AMP-increasing compounds alone or in combination with at least one meiosis-stimulating compound as active substances for the production of a pharmaceutical composition for the treatment of infertility in mammals.

7. Use according to claim 6, wherein the mammal is a human.

8. Use according to one of the claims 6 or 7, wherein the c-AMP-increasing compounds are used alone.

9. Use according to one of the claims 6, 7 or 8, wherein the c-AMP-increasing compound is selected from the group consisting of purines, unspecific PDE-inhibitors, specific PDE_3-inhibitor and synthetic membrane permeable cAMP.

10. Use according to one of the claims 6-9, wherein the c-AMP-increasing compound is hypoxanthine.

11. Use according to one of the claims 6, 7, 9 or 10 , wherein the meiosis-stimulating compound is FF-MAS.

12. Use of low dose c-AMP-increasing compounds alone or in combination with at least one meiosis-stimulating compound to increase the rate of fertilization in a mammal.

13. Use according to claim 12, wherein the mammal is a human.

14. Use according to one of the claims 12 or 13 to regulate the fertilization rate in fertilization culture media.

15. Use according to one of the claims 12-14, wherein the c-AMP-increasing compounds are used alone.

16. Use according to one of the claims 12-15, wherein the c-AMP-increasing compound is selected from the group consisting of purines, unspecific PDE-inhibitors, specific PDE_3-inhibitors and synthetic membrane permeable cAMP.

17. Use according to one of the claims 12-16, wherein the c-AMP-increasing compound is hypoxanthine.

18. Use according to one of the claims 12-14 or 16-17, wherein the meiosis-stimulating compound is FF-MAS.

19. Use of a low dose c-AMP-increasing compounds alone or in combination with at least one meiosis-stimulating compound for the administration to a germ cell.

20. Use according to claim 19, wherein the germ cell is an oocyte.

21. Use according to claim 19, wherein the germ cell is a spermatozoon.

22. Use according to one of the claims 19, 20 or 21, wherein c-AMP-increasing compounds are used alone.

23. Use according to one of the claims 19-22, wherein the c-AMP-increasing compound is selected from the group consisting of purines, unspecific PDE-inhibitors, specific PDE3-inhibitors and synthetic membrane permeable cAMP.

24. Use according to one of the claims 19-23, wherein the c-AMP-increasing compound is hypoxanthine.

25. Use according to one of the claims 19-21 or 23-24 wherein the meiosis-stimulating compound is FF-MAS.

26. A method of stimulating meiosis in a mammalian germ cell comprising administering *ex vivo* or *in vivo* or *in vitro* to a germ cell in need of such a stimulation an effective amount of low dose c-AMP-increasing compounds alone or in combination with at least one meiosis-stimulating compound.

27. A method according to claim 26, wherein the germ cell is an oocyte

28. A method according to claim 26, wherein the germ cell is a spermatozoon.

29. A method according to one of the claims 26-28, wherein c-AMP-increasing compounds are used alone.

30. A method according to one of the claims 28-31, wherein c-AMP-increasing compound is selected from the group consisting of purines, unspecific PDE-inhibitors, specific PDE_3-inhibitors and synthetic membrane permeable cAMP.

31. A method according to one of the claims 26-30 wherein the c-AMP-increasing compound is hypoxanthine.

32. A method according to one of the claims 26-28, 30 or 31, wherein the meiosis-stimulating compound is FF-MAS.

33. A pharmaceutical kit comprising a dosage unit for a c-AMP-increasing compound in low dose and a dosage unit of at least one meiosis-stimulating compound.